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Cloning and expression of cDNAs encoding plant V-ATPase subunits in the corresponding yeast null mutants

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Abstract

Complementation of yeast null mutants is widely used for cloning of homologous genes from heterologous sources. We have used this method to clone the relevant V-ATPase genes from lemon fruit and *Arabidopsis thaliana* cDNA libraries. The pH levels are very different in the vacuoles of the lemon fruit and the *A. thaliana*, yet both are the result of the activity of the same enzyme complex, namely the V-ATPase. In order to investigate the mechanism that enables the enzyme to maintain such differences in pH values, we have compared the subunit composition of the V-ATPase complex from both sources. Towards this end, we have constructed a cDNA library from lemon fruit and cloned it into a similar shuttle vector to the one of the *A. thaliana* cDNA library, which is commercially available. In this work, we report the cloning and expression of *VMA10* from both sources, two isoforms of the lemon proteolipid (*VMA3*) and the lemon homologue of yeast *VPH1*/*STV1* subunit, LEMAC.

Keywords: V-ATPase; Lemon fruit; *Arabidopsis thaliana*; Expression cloning; cDNA library

1. Introduction

Vacuolar ATPase (V-ATPase) belongs to a highly conserved family of proton-translocating ATPases present in all eukaryotic cells. They have been found in a variety of cellular organelles including lysosomes, endosomes, secretory and storage vesicles and plasma membrane, as well as on the vacuolar membrane. Each organelle has a specific requirement for its internal pH and membrane potential [1–4]. The internal pH of organelles of the vacuolar system is also variable and tightly regulated. While yeast vacuoles maintain an internal pH of about 5.5 it is assumed that the vacuoles of lemon fruit may have a pH as low as 2 [3]. Further evidence that the V-ATPase is regulated in plants is provided by the fact that the vacuolar pH can vary in different tissues of the same plant and in the same cell during the course of development or in response to changing environmental conditions. Crassulacean acid metabolism (CAM) plants are a classic example of vacuolar pH regulation. The pH of their leaf vacuoles fluctuates from pH 3 at night to pH 6 in the day [5]. During the maturation of citrus lemon fruit, the vacuolar pH of the juice sac cells declines from 5.5 to about 2. It has been proposed that a proton slip is one of the main factors in maintaining smaller ΔpH in different organelles of the vacuolar system [6]. Recently, differences in the composition of V-ATPase, isolated from vacuoles of distinct tissues of lemon fruit,

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were observed. The V-ATPase isolated from lemon fruit tissue was enriched in two polypeptides of 33–34 and 16 kDa that were hardly detected in the V-ATPase from lemon epicotyl tissue [7]. Moreover, the stalks of the juice sac V-ATPases appeared thicker than those of epicotyl V-ATPases in electron micrographs [8,9]. Cloning and sequencing of V-ATPase subunits from lemon fruit may reveal the clue for regulating the internal pH of organelles of the vacuolar system.

The V-ATPase consists of several polypeptides that are located in two major domains, a membrane peripheral domain (V1) and a membrane integral domain (V0). The V-ATPase holoenzyme from plant consists of up to 10 different subunits [10–12]. This is very close to the number of subunits found to be part of the mammalian and yeast V-ATPases [13]. Sequence information is available for seven of the plant V-ATPase subunits, i.e. subunits A, B, C, D, E, G and c [14]. Significant advances have already been made in animal and yeast V-ATPases and some other subunits have been cloned. For instance, a 100-kDa membrane integral subunit is present in the purified V-ATPase from most species studied to date, including yeast [15], bovine-coated vesicles [16] and red beets [17]. The lack of a 100-kDa subunit in some of the preparations of plant V-ATPases is particularly intriguing. Moreover, all plant subunit c isoforms cloned so far are homologues of the yeast *VMA3* gene [18]. In yeast and several other organisms, two additional genes encoding V-ATPase proteolipids have been identified, *VMA11* (coding for subunit c′) and *VMA16* (coding for subunit c″) [19]. It turned out that all three polypeptides are essential for the function of the V-ATPase holoenzyme in yeast. No such genes were identified in plants.

Yeast has proved to be an excellent model system to identify essential V-ATPase subunits. With the exception of *VPH1* and *STV1* that encode homologous proteins [20], all other V-ATPase subunit genes are present as a single copy in the yeast genome [21]. Disruption of each of the genes encoding essential V-ATPase subunits (except for *VPH1* or *STV1*) gave an identical phenotype in which yeast cells cannot grow at a pH higher than 7 and are sensitive to low and high calcium concentrations in the medium [21,22]. This is in contrast to the plant genome where genes have been reported to encode different isoforms of V-ATPase subunits (A, B, G and c) [23–25]. Therefore yeast provides a powerful screening system for the heterologous plant genes encoding V-ATPase subunits.

In this work, we have used the yeast system for isolating and expressing plant cDNAs encoding V-ATPase subunits. As a first step towards understanding the regulation of vacuolar pH in citrus fruit, we wanted to isolate the cDNAs encoding the subunits of V-ATPase from lemon fruit and compare them to those of *Arabidopsis thaliana*. Towards this end, we constructed a cDNA library from the juice sacs of lemon fruit. We anticipated that most of V-ATPases' unique properties would be attributed to the membrane sector. We therefore cloned the cDNA encoding subunit G (Vma10p) from lemon fruit, as well as from *A. thaliana,* using complementation of the yeast *VMA10* null mutant. Two isoforms of the proteolipid cDNA from lemon fruit were isolated by degenerate oligonucleotides and expressed in a yeast proteolipid-null mutant. In addition, we report the cloning and sequencing of the first cDNA encoding the plant homologues subunit of Vph1p/Stv1p from yeast.

2. Materials and methods

2.1. Construction of a cDNA library from lemon fruit

The total RNA was isolated from green lemons with a diameter of 2–3 cm. At this stage, the fruit is not sour and its pH is higher than 4.5. The young lemons were freshly picked from the tree, peeled from their skin by a sterile scalpel and frozen immediately in liquid nitrogen. The frozen lemon fruits were stored at −80°C until used for mRNA preparation. About 5 g of the fruits was crushed to dust in liquid nitrogen by a sterile mortar and pestle. For isolation of total RNA an SV Total RNA Isolation System (Promega) was used, and PolyATract mRNA Isolation System III (Promega) was used for the isolation of poly A containing mRNA. Reverse transcriptase (Gibco-BRL) was used for the synthesis of the first strand cDNA. And the Smart PCR (Clontech) kit was used for creating the second strand and amplification of the cDNA library by PCR. The re-

sulting cDNA was size fractionated on agarose gel and fragments higher than 2.0 kb were amplified by PCR with modified library primers containing *Not*I restriction sites at their ends. After *Not*I digestion the lemon cDNA was size fractionated on a DNA separating column (Clontech). The DNA from the first two fractions (above 2-kb fragments) was cloned into pFL61 yeast shuttle vector. The plasmids were transformed into *Escherichia coli* XL-2 Blue commercial competent cells (Stratagene). Colonies were grown on LB ampicillin plates over night, washed with LB ampicillin medium, some of the cell suspension was frozen and from the rest the plasmid DNA was isolated.

The *A. thaliana* cDNA library in pFL61 was obtained from the American Type Culture Collection.

2.2. Construction of specific primers

The primers used for the lemon fruit library cDNA amplification towards introduction of *Not*I sites at their ends were: the N′-primer 5′-TAT AGC GGC CGC TAC GGC TGC GAG AAG ACG ACA GAA G and the C′ terminal primer 5′-AAT TGC GGC CGC TTT TTT TTT TTT TTT.

The degenerate primers used for amplification of the proteolipid gene from the lemon cDNA were: (1) GC(GT) CC(CGT) TTC TTC GG(AC) TTC CT; (2) AGT AC(AT) GG(AGT) AT(CT) AAC CC(CT) AAG GC both used with the above mentioned C′-terminus primer; and (3) GCT TC(AT) GCG AA(AG) AT(AG) AG(AG) AT that was used with the above mentioned N′-terminus primer. With a second PCR using the first PCR products as templates and the N′- and C′-terminus primers as the reaction primers the full-length cDNA was obtained. Sequence analysis of several clones revealed two isoforms of the lemon fruit proteolipid.

The degenerate primers used for amplification of the *VPH1*/*STV1* lemon fruit cDNA homologue (LEMAC) were: (1) ATT CAC AC(AC) AT(AT) GA(AG) TTT GT(AG) CTT G; and (2) AC(AC) GCT TC(GT) TAC CT(GT) CGT CT(AG) TGG GC both used with the C′-terminus primer of the lemon fruit library in the PCR reactions. After sequence analysis specific antisense primers were used to amplify the N′-portion of the genes and a second PCR reaction as above gave the full size cDNA for the LEMAC gene.

2.3. Yeast strains

The wild-type yeast strain that was used in this work, is *Saccharomyces cerevisiae* W303 (*MATα trp1 ade2 leu2 his3 ura3*). The other strains used in this work are: Δ*VMA10* (*MATα trp1 ade2 leu2 his3 VMA10::URA3*), Δ*VMA3* (*MATα trp1 ade2 ura3 his3 VMA3::LEU2*), Δ*VMA11* (*MATα trp1 ade2 leu2 his3 VMA11::URA3*) and Δ*VPH1*/*STV1* (*MATα trp1 ade2 his3 VPH1::LEU STV1::URA*). The cells were grown in a YPD medium containing 1% yeast extract, 2% Bactopeptone and 2% dextrose. The medium was buffered by 50 mM MES and 50 mM MOPS, and the pH was adjusted by NaOH [22,26]. Agar plates were prepared by adding 2% agar to the YPD buffered medium at the given pH. Yeast transformation was performed as described previously [27], and the transformed cells were grown on minimal plates containing a 0.67% yeast nitrogen base, 2% dextrose, 2% agar and the appropriate auxotrophic substances.

2.4. Yeast transformation and functional complementation

Yeast library transformation was performed by the method of Ito et al. [27]. Plasmid Transformation was performed by a bench-top method according to Elble [28]. Selected clones were spread on YPD buffered at pH 7.5 plates to achieve the functional complementation. Measurements of quinacrine uptake were performed according to [29] using a fluorescent microscope (Nikon FXA).

2.5. DNA isolation from yeast

Yeast cells were grown in a selective medium to stationary phase. The cells were harvested by centrifugation for 2 min at 2500 rpm. The pellet was suspended in 100 μl of STET solution containing 50 mM Tris pH 8.0, 50 mM EDTA, 5% Triton X-100 and 8% sucrose. Glass beads (about 0.2 g) were added, and the suspension was vortexed for 20 min. Then, an additional 100 μl of STET were added, and the mixture was boiled for 3 min, cooled

for 1 min on ice, and centrifuged for 10 min at 13 000 rpm. A 100-μl amount was removed from the suspension and 50 μl of 7.5 M ammonium acetate was added. The mixture was incubated for 1 h in −20°C and centrifuged for 10 min at 13 000 rpm. A 100-μl amount of the suspension was removed to a fresh tube, 200 μl of cold ethanol was added, and the mixture was centrifuged for 30 min at 13–000 rpm. The pellet was washed with 70% ethanol and dissolved in 20 μl of 10 mM Tris and 1 mM EDTA pH 8.0.

3. Results

In order to investigate the underlying mechanism of the maintenance of a very low pH in the vacuole, we constructed a cDNA library from lemon fruit into the *Not*I site of a yeast shuttle vector pFL61. Because of the low pH of the fruit and the large amounts of irrelevant material, the total RNA was isolated from green lemons with a diameter of 2–3 cm. At this stage, the fruit is not sour and its pH is higher than 5. From the isolated poly A containing mRNA, full-length cDNAs were synthesized by reverse transcriptase (see Section 2). Double strand cDNA was obtained and amplified by PCR with specific primers for the N′- and C′-terminus of the mRNA (SMART PCR cDNA Construction kit from Clontech). A large amount of cDNA with size ranging from 0.6 to 5 kb DNA was obtained (Fig. 1A). The DNA was size-fractionated on agarose gel and the fragments above 2 kb were amplified by PCR with primers containing *Not*I restriction sites. After *Not*I digestion, the DNA was size-fractionated on a DNA separating column (Clontech) (Fig. 1B). Sixteen fractions of 35 μl each were collected and the cDNA from fractions one and two was ligated into pFL61 shuttle vector. The resulting cDNA library consisted of about 60 000 independent colonies containing 0.5–2.2-kb inserts. The lemon cDNAs were expressed in yeast mutants lacking the relevant V-ATPase subunit.

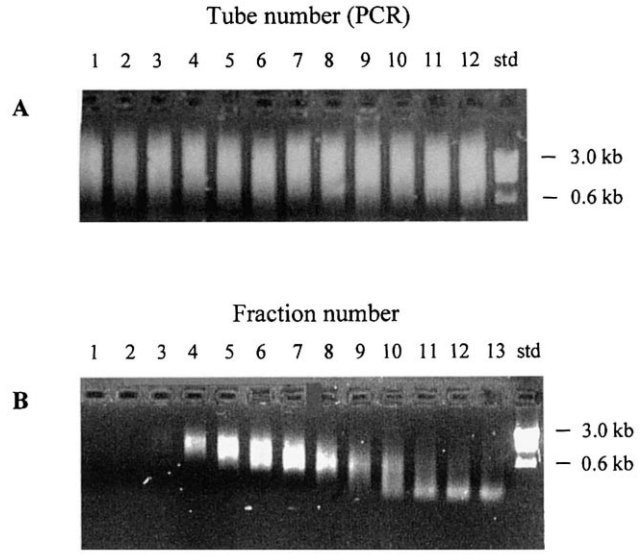

Fig. 1. The lemon fruit cDNA library. (A) 6 μg mRNA isolated from lemon fruit was used for first strand cDNA synthesis. Second strand synthesis and cDNA amplification was performed by PCR with specific primers (Smart PCR kit, Clontech) in 11 tubes containing 100 μl reaction mix each. A sample of 5 μl from each tube of the PCR reaction was electrophoresed on a 1.1% agarose gel (lanes 1–12). (B) Size fractionation of the cDNA from lemon fruit on a DNA separating column (Smart PCR kit, Clontech). A 3-μl sample of each fraction (35 μl) was electrophoresed on 1.1% agarose gel (lanes 1–13). DNA size markers (0.6, 1.1, 1.6, 2.2 and 3.0 kb) were loaded on the right side of the gels.

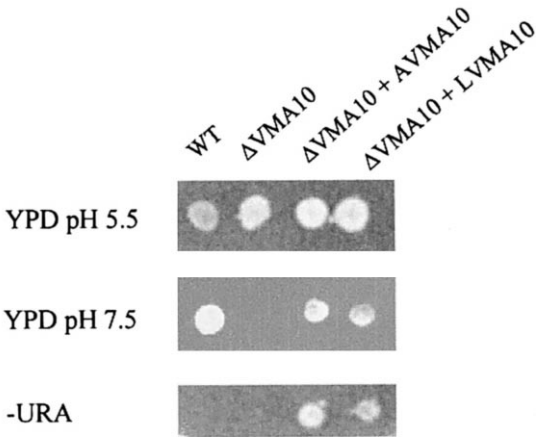

Fig. 2. Complementation of the yeast null mutant vma10::URA3/FoA with LVMA10 and AVMA10. The yeast null mutant vma10::URA3/FoA was transformed with the pFL61 shuttle vector carrying the lemon cDNA (LVMA10) or the *Arabidopsis thaliana* cDNA (AVMA10). Transformed yeast cells were grown on YPD medium buffered at pH 5.5, 7.5 and on minimal medium without uracil (−URA).

This approach of yeast functional complementation was used for isolating the cDNA encoding subunit G (Vma10p) from lemon and from the *A. thaliana* cDNA library in pFL61 (ATCC). Like any other mutants in which a gene encoding a V-ATPase sub-

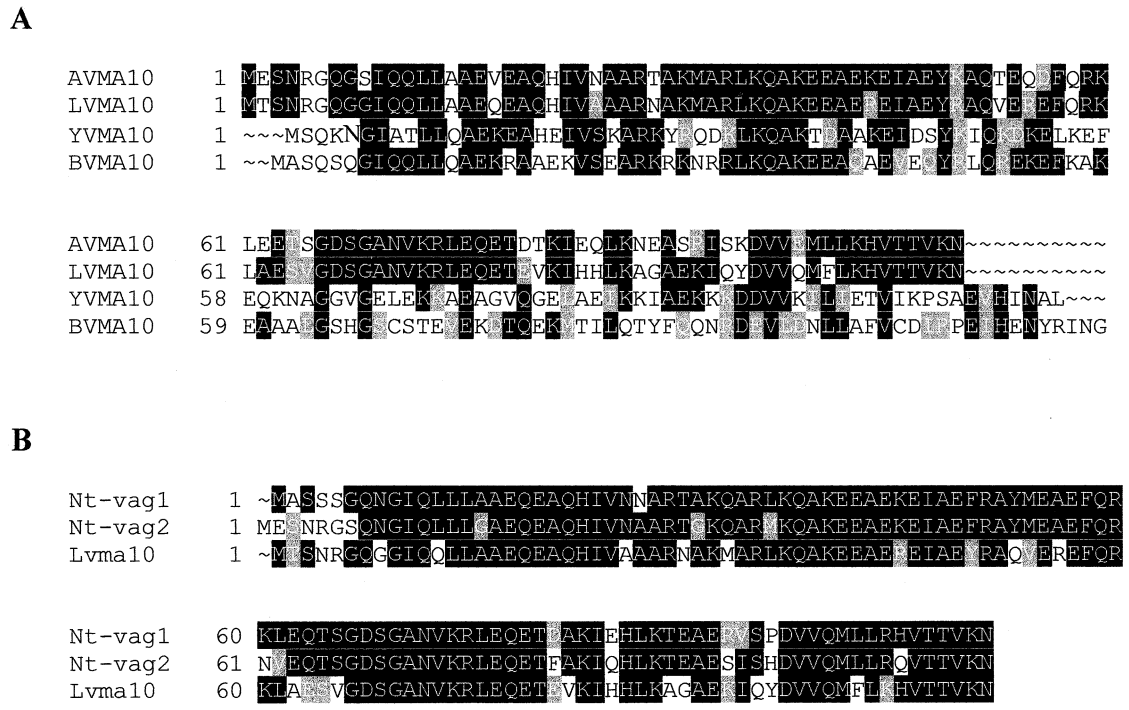

Fig. 3. Amino acid alignment of G subunits of V-ATPases from various species. (A) Subunit G protein sequences are from: *Arabidopsis thaliana* AVMA10 (AF181688) (this paper), *Citrus lemon* LVMA10 (AF184068) (this paper), *Saccharomyces cerevisiae* YVMA10 (U21240) and bovine BVMA10 (P79251). (B) Comparison of amino acid sequences of V-ATPase subunit G from tobacco (Nt-vag1 and Nt-vag2) (AJ005899 and AJ005900) and lemon (LVMA10) (AF184068) (this paper). The sequences were aligned using the pileup program. Boxshade program was used for visualizing the results.

unit has been disrupted, the yeast null mutant *vma10::URA3/FoA* failed to grow at pH 7.5 [22]. After transformation with the plant cDNA library, yeast cells that were able to grow in minimal medium without uracil were spread on YPD medium buffered at pH 7.5. Plasmids were purified from colonies grown on a buffered medium at high pH, and following amplification in *E. coli*, were used for second transformation of the yeast null mutant. Under these conditions, only yeast carrying the *VMA10* homolog from the plant was able to grow on YPD pH 7.5 and in minimal medium without uracil ([29] and Fig. 2). The new cDNAs from lemon (LVMA10) and from *A. thaliana* (AVMA10) were sequenced. The deduced amino acid sequences of VMA10 from *A. thaliana* and lemon shared 77% identity (Fig. 3A). Amino acid alignment of VMA10 from plants with the V-ATPase subunit G characterized in yeast (YVMA10) and bovine (BVMA10) showed that they are more similar to the bovine homologue (37% identity) than to the yeast (34% identity) (Fig. 3A). In general, there are few amino acids that are conserved between all of them. Most of these residues are located in the N-terminal half of the proteins. We assume that these amino acids are essential for the activity of the protein. High homology of the lemon VMA10 was found in two isoforms of subunit G isolated recently from *Nicotiana tabacum* ([24] and Fig. 3B). This implies that the subunit G of V-ATPase is conserved among plants.

The proteolipid, which is a very conserved protein [2], was isolated from the lemon fruit cDNA library by PCR. Degenerated oligonucleotides were designed according to relatively conserved regions of proteolipids from different plant species. The clones that were obtained were sequenced. Sequence analysis of these clones revealed that there are at least two isoforms encoding the proteolipid from lemon. The two isoforms, LPL-I and LPL-II, shared 77% identity in their nucleotide sequence. The clones were different in their codon usage and in their 5′- and 3′-untranslated regions. From the deduced amino acid sequen-

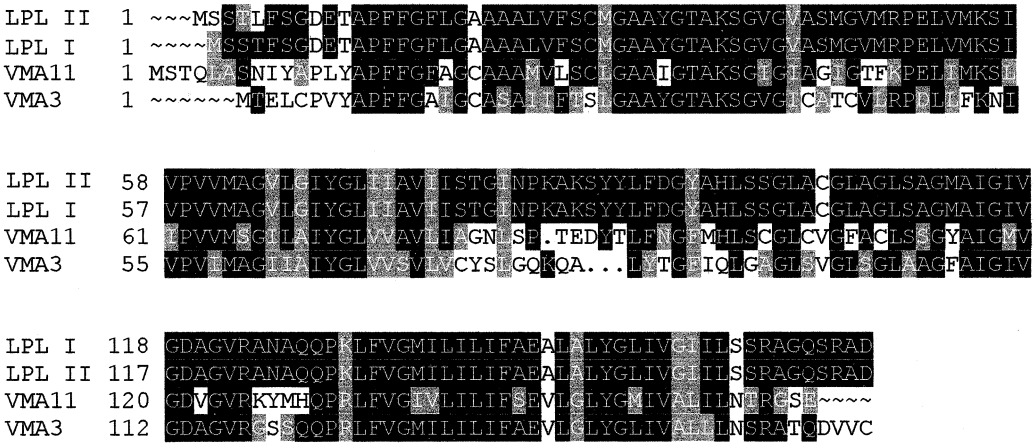

Fig. 4. Comparison of deduced amino acid sequences of LPL-I and LPL-II with the yeast proteolipids VMA3 (NP_010887) and VMA11 (NP_015090). The proteins were aligned using the pileup program. Boxshade program was used for visualizing the results.

ces, which were 98% identical, we have concluded that LPL-I and LPL-II coded for the same subunit. In fact, there is one additional amino acid (L) in the N′-terminus of the LPL-II isoform (Fig. 4). These results provide evidence for a small multigene family encoding the V-ATPase proteolipid from lemon.

The lemon proteolipid shares 60% identity with the yeast proteolipid (Vma3p). Interestingly, similar percent homology was found with the yeast Vma11p. Vma11p and Vma3p share 56% identity (Fig. 4). To determine whether the lemon proteolipid isoforms were capable of restoring V-ATPase activity to the corresponding yeast null mutants, lemon cDNA clones, LPL-I and LPL-II, were expressed in yeast *VMA3::LEU*. We found that only LPL-II partially rescued the phenotype of inability to grow at physiological pH (Fig. 5). To determine if vacuole acidification is restored in the rescued Δ*VMA3* yeast, cells were washed with a dilute solution of the pH probe quinacrine. Fluorescent signals were readily detected in vacuoles of wild-type, but not in the transformed yeast cells (data not shown). We think that the vacuoles failed to accumulate enough quinacrine because of the very low V-ATPase activity in the yeast cells containing the lemon proteolipid. In light of the high homology to Vma11p from yeast and the fact that a plant homolog to Vma11p has not been found yet, we tested whether LPL-I and LPL-II functionally complement the Δ*VMA11* yeast. The two isoforms of the lemon proteolipid were expressed in the Δ*VMA11* null mutants. None of the isoforms complement the growth on YPD medium buffered at pH 7.5, of the yeast null mutant cells (data not shown).

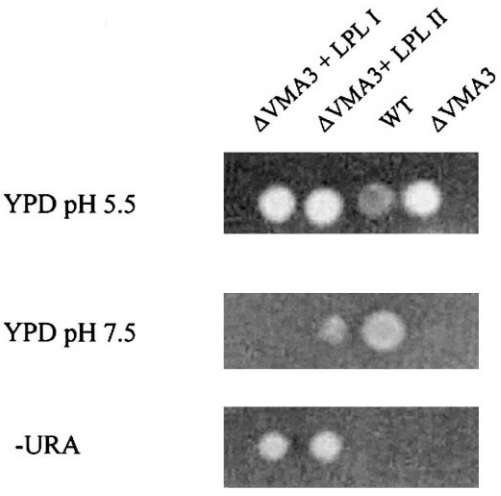

Fig. 5. The lemon proteolipid isoform LPL-II partially complements the yeast null mutant vma3::LEU. The yeast null mutant vma3::LEU was transformed with the pFL61 shuttle vector carrying LPL-I or LPL-II cDNAs. Transformed yeast cells were grown on YPD medium buffered at pH 5.5, 7.5 and on minimal medium without uracil (−URA).

For isolating the cDNA encoding the lemon homolog to Vph1p/Stv1p subunit of V-ATPase from yeast, we used degenerate primers designed according to relatively conserved sequences from yeast and mammalian genes and also from plant ESTs, found in the GenBank. The partial PCR products were sequenced and the deduced amino acid sequences were similar to those of Vph1p/Stv1p subunits in the data base. Complete cDNA of the lemon homolog was

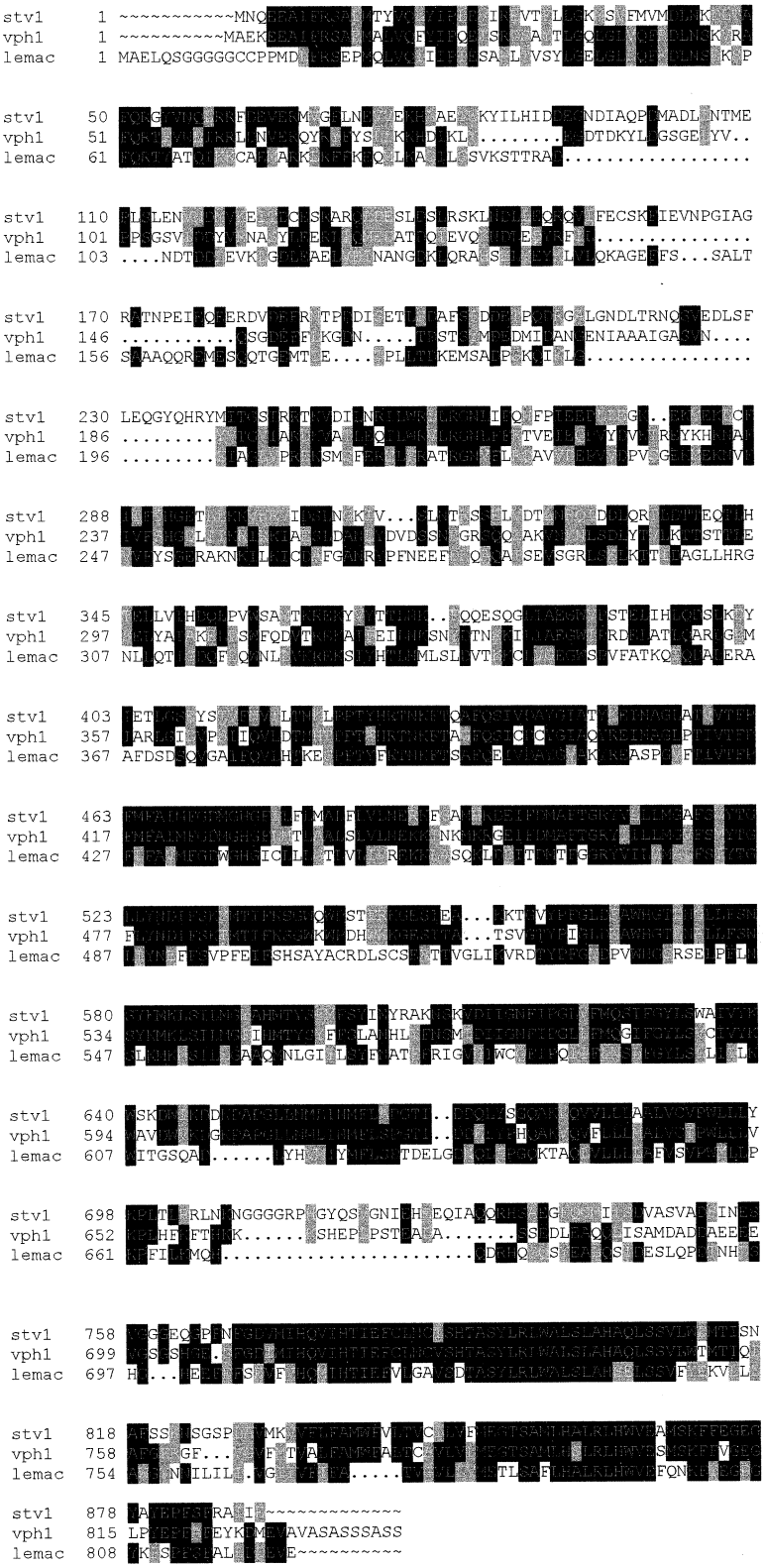

Fig. 6. Amino acid alignment of the lemon V-ATPase subunit AC115 (Lemacp) with the two homologues from yeast Vph1p (p32563) and Stv1p (p327296). The proteins were aligned using the pileup program. Boxshade program was used for visualizing the results.

obtained by PCR on the partial clones, with the primers corresponding to the N′- and C′-terminus of the lemon cDNA library (see Section 2). Search in GenBank revealed that this is the first cDNA encoding the Stv1p/Vph1p subunit isolated from plants. The lemon protein (LEMAC) showed 43 and 37% identity to Stv1p and Vph1p, respectively, while the two homologs from yeast share 60% identity (Fig. 6). In order to investigate the function of this protein, we cloned it into the shuttle vector pFL61, in an attempt to express it in a yeast null mutant. Because Stv1p and Vph1p encode homologous proteins in yeast, we used the double mutant Δ*stv1*/*vph1* for complementation. So far, the lemon homolog was not capable of restoring the growth of the V-ATPase mutant at pH 7.5 (data not shown).

4. Discussion

V-ATPases normally operate far from thermodynamic equilibrium and are therefore considered to be under kinetic regulation. In lemon fruits, the transtonoplast pH gradient is close to the calculated maximum for a V-ATPase operating at thermodynamic equilibrium (pH 4–5) assuming an H^+/ATP stoichiometry of 2 [30,31]. The energy-consuming organelles may utilize coupling modulation as part of the regulation of their bioenergetic processes. The apparent variable stoichiometry of proton to ATP coupling in V-ATPases led to the suggestion that V-ATPase is a proton pump controlled by a slip [6]. It is logical to assume that in those organelles (such as lemon vacuoles), in which internal pH is maintained at pH 2 or below, most of the slip was canceled, and the degree of coupling was elevated to the optimal level. An intriguing possibility of controlling the coupling ratio of the enzyme is the modification of the subunit composition of the holoenzyme. This could be achieved by changes of the copy number of subunits in the holoenzyme complex or by incorporation of new or specialized subunits. We assumed that the key to the control of the coupling ratio of the enzyme lies in the membrane sector of the enzyme. In this study, we described the cloning and sequencing of subunit G, the proteolipid and the *VPH1*/*STV1* homolog from the lemon fruit cDNA library we constructed. The function of these subunits was studied in the yeast *S. cerevisiae*. Expression of plant cDNAs encoding V-ATPase subunits in yeast null mutants is very useful for elucidating specific properties of the plant V-ATPase subunits.

Subunit G exhibits significant sequence similarity to the F-ATPase b-subunit, although it is shorter and does not contain an apparent transmembrane domain at its N-terminus [32]. In analogy to the F-ATPase b-subunit, subunit G is a good candidate for participating in the peripheral V-ATPase stalk which might be involved in coupling of ATP-hydrolysis and H^+-transport. Subunit G was isolated from the lemon fruit V-ATPase by functional yeast complementation. In order to compare the amino acid sequence and properties of the protein to another plant species where the vacuoles are not hyperacidified, subunit G was isolated from the *A. thaliana* cDNA library (ATCC). The proteins from lemon and *A. thaliana* show high homology (77%) throughout their amino acid sequence (Fig. 3A), and both restored the wild-type phenotype of the yeast *VMA10* null mutant (Fig. 2). The lemon VMA10 shares high homology to two related subunits G that were recently identified in *Nicotiana tabacum* (Fig. 3B). The two clones were functional in yeast vma10 null mutant [24]. These data implicate that Vma10p is conserved among plants and shares similar activities within the cell of different organisms.

Subunit c (the proteolipid) is the main component of the membrane (V0) domain. It is a highly hydrophobic protein, containing four membrane-spanning domains [33,34] and is suggested to be directly involved in H^+-transport. Subunit c genes have been cloned from several plants and for most of them small gene families have been detected: Four different isoforms have been cloned in *A. thaliana* [25] and *Av. sativa* [35], while two subunit c isoforms have been identified in *G. hirsutum* [36], *M. crystallinum* [37] and *Zea mays* [38]. We report the cloning of two isoforms, LPL-I and LPL-II, of the proteolipid from lemon fruit. A common feature of all subunit c isoforms is a high degree of sequence similarity in the coding regions, while they differ significantly in the non-coding regions. The two cDNAs from lemon encode near-identical proteins which differ in one additional amino acid present in the N′-terminus of the LPL-II isoform (Fig. 4). Regulation of V-ATPase activity by expression of different subunit isoforms in

various tissues or under certain environmental conditions would be an intriguing possibility. Moreover, multiple genes can provide isoforms with unique properties and levels of expression to satisfy the specialized requirement of H^+ pumping activity for different tissues, cell types, or subcellular organelles. If so, then one interesting question is whether both or just one of the lemon proteolipids is responsible for the hyperacidification of the fruit vacuoles. The LPL-II isoform partially complements the yeast Δ*vma3* phenotype of inability to grow on YPD pH 7.5 (Fig. 5). This is despite the fact that the plant subunit was not capable of restoring the vacuole acidification visualized by fluorescent assay. It may be assumed that if the proteolipid isoform causes hyperacidification in the lemon fruit vacuoles, expressing it in yeast may slow the growth rate of the cells, and the vacuole acidification could not be detected. Further work is needed to confirm this hypothesis. All plant subunit c isoforms cloned so far are homologous of the yeast *VMA3* gene that is essential for the production of an active V-ATPase holoenzyme. However, in yeast two additional proteolipid genes have been identified [18,21], *VMA11* (coding for subunit c′) and *VMA16* (coding for subunit c″). The sequence similarity of Vma11p and Vma3p is 60%, while comparison of the Vma16p sequence with the sequences of Vma3p and Vma11p shows a similarity of about 30%. Vma3p and Vma11p contain four transmembrane helices, while in Vma16p five transmembrane helices are present [18,21]. Interestingly, the lemon proteolipid isoforms show similar homology (about 60%) to both Vma3p and Vma11p (Fig. 4). One explanation for this phenomenon is that the multiple isoforms compensate for the absence of the additional proteolipids found in yeast, and regulate the enzyme activity. However, proteins exhibiting structural similarity to c″ have recently been identified in the worm *Caenorhabditis elegans* and *A. thaliana* by sequence comparison [39]. If c, c′ and c″ really are present in the plant V-ATPase membrane domain, changes of the coupling ratio of the V-ATPase might not only be due to the expression of different c-subunit isoforms, but also to the modification of the stoichiometry of c:c′:c″ in the V0 domain.

Polypeptides of 95–115 kDa have been demonstrated to co-purify with V-ATPase subunits in several, but not all plant species. It is unclear whether these polypeptides are analogues to the Vph1/Stv1 proteins of the yeast V-ATPase. Both polypeptides do not exhibit sequence similarity to F-ATPase subunits and seem to be unique to the V-ATPase. We wondered whether a plant homolog to Vph1p/Stv1p was present in lemon fruit, and if so what the specific role of such a polypeptide was in the fruit V-ATPase. In trying to answer this question, we isolated from the lemon fruit cDNA library the first cDNA encoding a homolog to the Vph1p/Stv1p subunit from plants (LEMAC). The protein showed higher homology to Stv1p subunit of V-ATPase than to Vph1p (Fig. 6). During the preparation of this manuscript, an additional search in the GenBank revealed the sequences of two genes that encode related proteins of about 100 kDa (AC006841 and AL035679) from *A. thaliana*. Thus it seems that plants, like yeast, have two alternative genes encoding the AC115 subunit, as well. The deduced sequence of the 100–116-kDa protein from yeast or mammals shares a structure with an extensive N-terminal hydrophilic domain and a C-terminal domain with up to seven putative transmembrane segments [20,40]. Site-directed mutagenesis of residues in the putative transmembrane segments in Vph1p indicates that this subunit is required for assembly and proton translocation [41,42]. Future studies on the structural modifications of the membrane domain may shed light on the control mechanisms of internal pH in the vacuolar system.

Acknowledgements

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References

[1] N. Nelson, J. Bioenerg. Biomembr. 21 (1989) 553–571.

[2] N. Nelson, Biochim. Biophys. Acta 1100 (1992) 109–124.
[3] L. Taiz, J. Exp. Biol. 172 (1992) 113–122.
[4] M. Forgac, Soc. Gen. Physiol. Ser. 51 (1996) 121–132.
[5] U. Luttge, New Phytol. 106 (1987) 593–629.
[6] Y. Moriyama, N. Nelson, in: W.D. Stein (Ed.), The Ion Pumps, Structure, Function and Regulation, Alan R. Liss, New York, l988, pp. 387–394.
[7] M.L. Muller, U. Irkens-Kiesecker, B. Rubinstein, L. Taiz, J. Biol. Chem. 271 (1996) 1916–1924.
[8] M.L. Muller, U. Irkens-Kiesecker, D. Kramer, L. Taiz, J. Biol. Chem. 272 (1997) 12762–12770.
[9] M.L. Muller, M. Jensen, L. Taiz, J. Biol. Chem. 274 (1999) 10706–10716.
[10] J.M. Ward, H. Sze, Plant Physiol. 99 (1992) 170–179.
[11] F.M. DuPont, P.J. Morrissey, Arch. Biochem. Biophys. 294 (1992) 341–346.
[12] M. Hosaka, K. Kanayama, K. Shiratake, S. Yamaki, Phytochemistry 36 (1994) 565–567.
[13] N. Nelson, D.J. Klionsky, Experientia 52 (1996) 1101–1110.
[14] R. Ratajczak, Biochim. Biophys. Acta 1465 (2000) 17–36.
[15] P.M. Kane, T.H. Stevens, J. Bioenerg. Biomembr. 24 (1992) 383–393.
[16] I. Adachi, K. Puopolo, N. Marquez-Sterling, H. Arai, M. Forgac, J. Biol. Chem. 265 (1990) 967–973.
[17] R.V. Parry, J.C. Turner, P.A. Rea, J. Biol. Chem. 264 (1989) 20025–20032.
[18] T.H. Stevens, M. Forgac, Annu. Rev. Dev. Biol. 13 (1997) 779–808.
[19] R. Hirata, L.A. Graham, A. Takatsuki, T.H. Stevens, Y. Anraku, J. Biol. Chem. 272 (1997) 4795–4803.
[20] M.F. Manolosn, B. Wu, D. Proteau, B.E. Taillon, B.T. Roberts, M.A. Hoyt, E.W. Jones, J. Biol. Chem. 269 (1994) 14064–14074.
[21] N. Nelson, W.R. Harvey, Phys. Rev. 79 (1999) 361–385.
[22] H. Nelson, N. Nelson, Proc. Natl. Acad. Sci. USA 87 (1990) 3503–3507.
[23] U. Luttge, R. Ratajczak, Adv. Bot. Res. 25 (1997) 253–296.
[24] D. Rouquie, C. Tournaire-Roux, W. Szponarski, M. Rossignol, P. Doumas, FEBS Lett. 437 (1998) 287–292.
[25] I.Y. Perera, X. Li, H. Sze, Plant Mol. Biol. 29 (1995) 227–244.
[26] T. Noumi, C. Beltrán, H. Nelson, N. Nelson, Proc. Natl. Acad. Sci. USA 88 (1991) 1938–1942.
[27] H. Ito, Y. Fukuda, K. Murata, A. Kimura, J. Bacteriol. 153 (1983) 163–168.
[28] R. Elble, BioTechniques 13 (1992) 18–20.
[29] L. Supekova, F. Supek, N. Nelson, J. Biol. Chem. 270 (1995) 13726–13732.
[30] A.L. Schmidt, D.P. Briskin, Arch. Biochem. Biophys. 301 (1993) 165–173.
[31] P.A. Rea, D. Sanders, Physiol. Plant. 71 (1987) 131–141.
[32] L. Supekova, M. Sbia, F. Supek, Y. Ma, N. Nelson, J. Exp. Biol. 199 (1996) 1147–1156.
[33] M. Mandel, Y. Moriyama, J.D. Hulmes, Y.C.E. Pan, H. Nelson, N. Nelson, Proc. Natl. Acad. Sci. USA 85 (1988) 5521–5524.
[34] H. Nelson, N. Nelson, FEBS Lett. 247 (1989) 147–153.
[35] S. Lai, J.C. Watson, J.N. Hansen, H. Sze, J. Biol. Chem. 266 (1991) 16078–16084.
[36] M.P. Hasenfratz, C.L. Tsou, T.A. Wilkins, Plant Physiol. 108 (1995) 1395–1404.
[37] R. Low, B. Rockel, M. Kirsch, R. Ratajczak, S. Hortensteiner, E. Martinoia, U. Luttge, T. Rausch, Plant Physiol. 110 (1996) 259–265.
[38] R. Viereck, M. Kirsch, R. Low, T. Rausch, FEBS Lett. 384 (1996) 285–288.
[39] T. Oka, R. Yamamoto, M. Futai, J. Biol. Chem. 272 (1997) 24387–24392.
[40] S.B. Peng, B.P. Crider, X.S. Xie, D.K. Stone, J. Biol. Chem. 269 (1994) 17262–17266.
[41] X.H. Leng, M.F. Manolson, Q. Liu, M. Forgac, J. Biol. Chem. 271 (1996) 22487–22493.
[42] X.H. Leng, M.F. Manolson, M. Forgac, J. Biol. Chem. 273 (1998) 6717–6723.